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Epstein–Barr Virus Nuclear Antigen-1 Binds to Nuclear Transporter Karyopherin α1/NPI-1 in Addition to Karyopherin α2/Rch1

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We searched for cellular proteins that interact with Epstein–Barr (EBV) virus nuclear antigen-1, which is a latent EBV origin-binding protein detected in all EBV latently infected cells and essential for maintenance of the latent EBV genome, by a yeast two-hybrid screening of a B lymphocyte cDNA library in this study. Interaction of polypeptides synthesized from three selected cDNA clones with EBNA-1 proteins was confirmed *in vitro* using their glutathione-*S*-transferase-fusion polypeptides and by coimmunoprecipitation analyses of B cell extracts with anti-EBNA-1 monoclonal antibodies and monospecific antibodies against cellular proteins of interest. We report the following: (i) Karyopherin α (karyopherin α1, hSRP1, and NPI-1), an adaptor subunit of nuclear localization signal receptors, which direct proteins to the nuclear pore, interacted with EBNA-1. (ii) EBNA-1 proteins endogenous in the B cell line Raji of Burkitt lymphoma origin bound to another adaptor protein, karyopherin α2 (hSRP1α, hRch1), interactions of which to recombinant EBNA-1 polypeptides were previously reported. (iii) Nearly 90% of all the cDNA clones examined was p32 (SF2-associated P32, p32/TAP, and gC1q-R), and endogenous EBNA-1 proteins in the Raji cells bound to p32, a potential of which to affect localization of EBNA-1 in transfected Vero cells has been recently suggested. These results suggest that EBNA-1, which has the unique NLS containing Lys–Arg and overlapping with one of the phosphorylation domains, is recognized and transported to the nuclei by these two distinct karyopherin α proteins, which are differentially expressed in different cell types, implying a regulatory localization system for EBNA-1.

INTRODUCTION

Epstein–Barr virus (EBV) nuclear antigen-1 and the recently identified BARF0 are the only two viral-encoded proteins that are expressed in type I latency (the EBNA-1-only program) of EBV (Kieff, 1996; Fries *et al.,* 1997). EBNA-1 is also expressed in a subpopulation of normal B cells latently infected with EBV (Chen *et al.,* 1995; Miyashita *et al.,* 1997), although in the resting EBV-positive normal B lymphocytes EBNA-1 mRNA from the *Bam*HI Q promoter, Qp, was not detected (Davenport and Pagano, 1999). EBNA-1 plays an essential role(s) in the maintenance/segregation of latent EBV plasmid DNA and possibly in EBV DNA partitioning to host chromosomes (Aiyar *et al.,* 1998; Lee *et al.,* 1999). EBNA-1 transactivates the promoter(s) Wp/Cp for a polycistronic message of all six EBNAs in type III latency (Rickinson and Kieff, 1996) and negatively regulates Qp during latency I in which none of the other EBNAs is transcribed (Kieff, 1996; Davenport and Pagano, 1999). EBNA-1 also resembles "RGG" RNA binding proteins (Snudden *et al.,* 1994). In addition, it was reported that EBNA-1 antisense oligodeoxyribonucleotides inhibited proliferation of EBV-immortalized cells (Roth *et al.,* 1994), EBNA-1 expression in transgenic mice induced lymphoma (Wilson *et al.,* 1996), EBNA-1 increased the tumorigenicity and metastatic capability of a nasopharyngeal carcinoma cell line (Sheu *et al.,* 1996), and EBNA-1 expression was associated with enhanced expression of CD25 in a Hodgkin cell line (Kube *et al.,* 1999).

It is well established that EBNA-1 is localized in the nucleus; however, whether and how a cellular mechanism controls its subcellular or subnuclear localization, which might play a role in the regulation or modulation of its functions, are only poorly understood. EBNA-1 is phosphorylated at serine residues (Hearing and Levine, 1985; Polvino-Bodnar *et al.,* 1988; Frappier and O'Donnell, 1991), yet a functional role(s) of this phosphorylation remains largely unknown. No enzymatic activity has been associated with EBNA-1 (Frappier and O'Donnell, 1991), and EBNA-1 functions in the absence of other EBV proteins during the latency of type I and probably types II and III. Therefore identification and characterization of cellular proteins that interact with EBNA-1 protein are crucial to elucidate the molecular mechanisms of EBNA-1's regulatory function(s). It has been suggested that EBNA-1 proteins bind to p32 [also known as SF2-associated p32, p32/TAP (HIV Tat-associated protein), and gC1q-R] (Wang *et al.,* 1997; Chen *et al.,* 1998), which is

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transcriptionally active and might play a role as the cellular coactivator that bridges EBNA-1 to TFIIB (Wang *et al.,* 1997; Yu *et al.,* 1995). Recently, it has also been reported that EBNA-1 binds to human RPA (hSSB), the replicative single-strand DNA binding protein that interacts with a variety of cellular and viral proteins (Zhang *et al.,* 1998), and to EBP2 (EBNA-1 binding protein 2) (Shire *et al.,* 1999).

In the present study, we have searched for cellular proteins that bind to EBNA-1 using an interaction trap/two-hybrid method (Chien *et al.,* 1991) of a human B lymphocyte cDNA library. Interactions of the two-hybrid selected cDNA proteins with EBNA-1 were directly analyzed using the glutathione-*S*-transferase–cDNA clone fusion polypeptides and specific antibodies against proteins of interest. Furthermore we analyzed coimmunoprecipitation of the cellular proteins using anti-EBNA-1 monoclonal antibodies.

In this report, we show that EBNA-1 interacts with karyopherin α protein [also known as karyopherin α1/hSRP1/NPI-1 (Mattaj and Englmeier, 1998)], referred to as NPI-1 hereafter, implying that the nuclear transport of EBNA-1 is mediated by this adaptor subunit of the nuclear import receptor. The karyopherin α adaptor proteins heterodimerize with karyopherin β/importin β proteins through the 41-aa importin-β-binding (IBB) domain, forming nuclear import receptors. The nuclear import receptors transport karyophilic proteins to the nucleus via the nuclear pore complex and release them upon association with RanGTP. We show also that endogenous EBNA-1 proteins in B cells interact with another adaptor subunit called karyopherin α2 [known also as hSRP1α/NPI-3/hRch1 (Mattaj and Englmeier, 1998)], referred to as Rch1 here. The significance of EBNA-1 interaction with these two discrete karyopherin αs is discussed.

RESULTS

Search for EBNA-1-interacting cellular proteins by interaction trap system and *in vitro* binding of EBNA-1 proteins to GST-fusion polypeptides of cellular cDNA clones

For a yeast two-hybrid system (Chien *et al.,* 1991) to screen cellular proteins that interact with EBNA-1 proteins, we constructed a truncated form of bait using the EBNA-1 deletion mutant recombinant DNA, p205, which has a deletion of 700 $\pm$ 20 bp from the Gly-Gly-Ala repeat region of 717 bp in the B-95 strain (Yates *et al.,* 1985). The Gly–Gly–Ala repeat domain inhibits the antigen processing and MHC class-I-restricted presentation via the ubiquitin/proteasome-dependent protein degradation of EBNA-1 (Levitskaya *et al.,* 1995, 1997), but the repeat domain is dispensable for the other functions of EBNA-1. The triplet repeat domain-deleted form of EBNA-1 was more efficient as two-hybrid bait than the aa 451–641 segment of EBNA-1 initially used in this study. We obtained 36 yeast colonies, which were dependent on EBNA-1 synthesized in the yeast cells in both assays of β-galactosidase and HIS3 expression. From these two-hybrid screening positive yeast colonies, cDNA clones were extracted and subcloned. We analyzed base sequences of the 36-cell cDNA clones and carried out a computer-aided homology search of the base sequences using BLASTN 1.4.11 (to be published elsewhere). To test whether polypeptides synthesized from the cDNAs of interest bind to EBNA-1 *in vitro*, glutathione-*S*-transferase fusion polypeptides were constructed (Fig. 1B) and transfected to *Escherichia coli* BL21 strain for expression. The retention of EBNA-1 proteins from cell lysates of Raji, the EBV latency type III cell line of Burkitt lymphoma origin, by the GST-fusion proteins that had been bound on glutathione–Sepharose 4B beads, was examined.

One interaction-trap-positive cDNA was NPI-1, and its GST-fusion bound to EBNA-1 in the presence and absence of B cell lysates

The 502-base sequence of the cDNA 3–90 was in agreement with the 424- to 925-bp region of hSRP1 [GenBank, Accession No. S75295; identities = 498/502 (99%)]. In addition, the reactivity of the GST-fusion protein of the cDNA clone 3-90 (Fig. 1B), referred to as GST-3-90 hereafter, to NPI-1-specific antibodies was confirmed by Western blot analysis (Fig. 2, lanes 7 and 8). These results indicate that the cDNA 3-90 is a cDNA of NPI-1. The protein retention assay of proteins in Raji cell lysate by GST-3-90 polypeptides on the glutathione beads demonstrated that the GST-3-90 polypeptides bound to EBNA-1 proteins in the Raji cells (Fig. 2, lane 5).

We next addressed the question of whether the interaction of the GST-3-90 and EBNA-1 proteins was mediated by other proteins in the Raji cell lysates. The EBNA-1 mutant EBNA-1(ΔGA), which is deficient in the Gly-Gly-Ala repeat region (Fig. 1A), is exactly the same truncated form as that employed in the two-hybrid screening bait in this study. The expressed EBNA-1(ΔGA) polypeptide was 58 kDa as determined from immunoblot analysis, larger than its predicted size, as in the case of the full-size EBNA-1 protein with apparent and predicted sizes of 78 and 56 kDa, respectively (Polvino-Bodnar *et al.,* 1988). The EBNA-1(ΔGA) polypeptides were incubated with the glutathione-Sepharose 4B bead-attached GST-3-90 fusion proteins. All proteins trapped by GST-3-90 polypeptides were eluted by adding the reduced form of glutathione and analyzed by Western blotting. A faint but clear 58-kDa band corresponding to the EBNA-1(ΔGA) and a smaller broadband, probably its proteolytic product, were detected (Fig. 2, lane 9), indicating that the NPI-1 polypeptides were capable of interacting with the

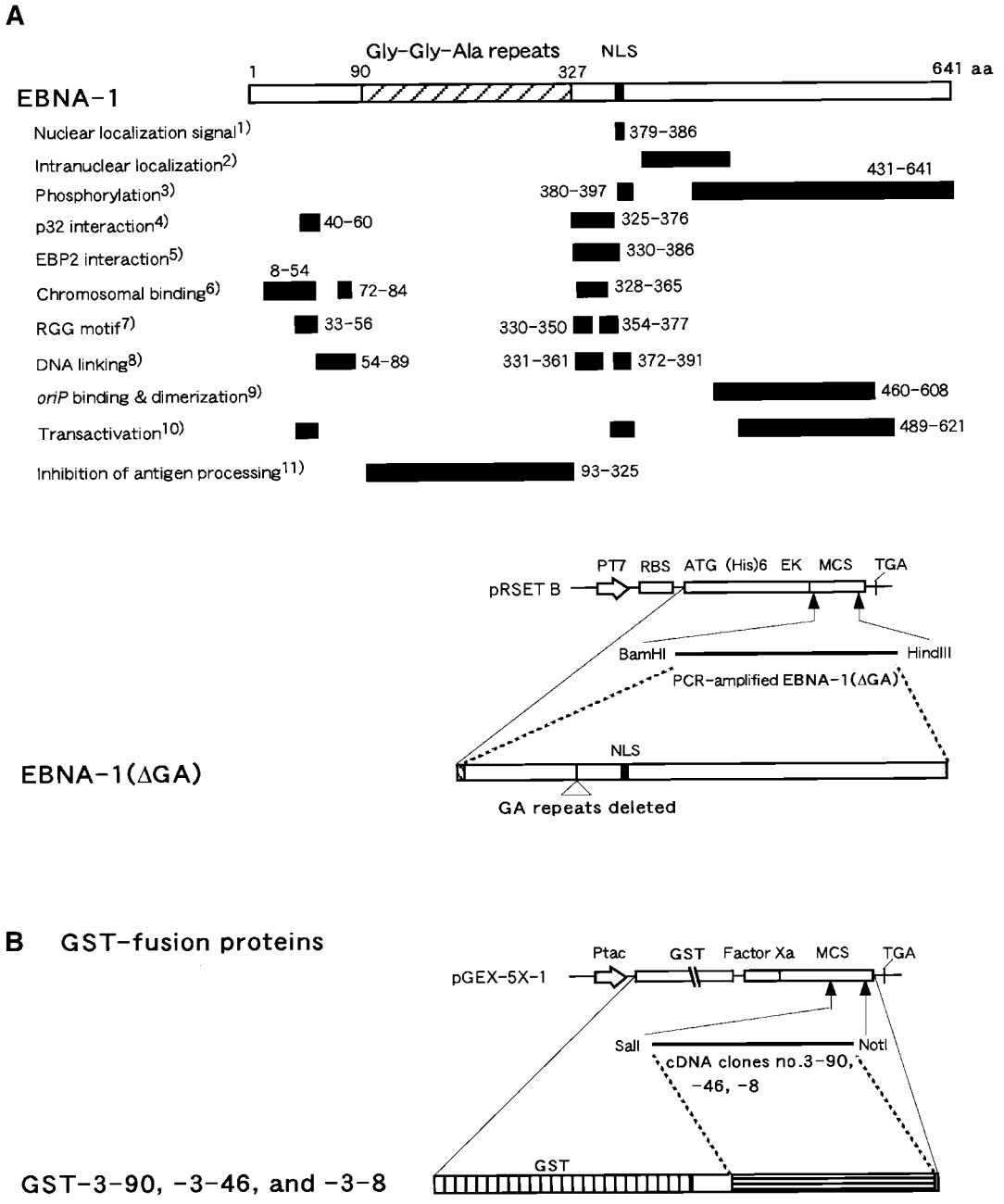

FIG. 1. Structure of EBNA-1 and construction of EBNA-1(ΔGA) and glutathione-*S*-transferase-fusion polypeptides of interaction trap-positive cDNA clones. The Gly–Gly–Ala repeat region is shown as a hatched box within the open bar representing the EBNA-1 gene of 641 aa at the top, and the functional domains of EBNA-1 are delineated by solid bars underneath (A). The diagram was drawn based on the following references which are indicated in each (A) (1) Ambinder *et al.* (1991); (2) Polvino-Bodnar *et al.* (1988); (3) Hearing and Levine (1985), Polvino-Bodnar *et al.* (1988), Polvino-Bodnar and Schaffer (1992); (4) Wang, Y. *et al.* (1997); (5) Shire *et al.* (1999); (6) Marechal *et al.* (1999); (7) Snudden *et al.* (1994); (8) Mackey *et al.* (1995); (9) Inoue *et al.* (1991), Chen *et al.* (1993); (10) Polvino-Bodnar *et al.* (1988), Yates and Camiolo (1988); and (11) Levitskaya *et al.* (1995, 1997). EBNA-1(ΔGA) was constructed by inserting the PCR-amplified p205 EBNA-1 sequence, which carries a 700 ± 20-bp deletion at the site of the Gly–Gly–Ala repeats of 717 bp (Yates *et al.,* 1985), into the expression vector pRSETB on the bottom (A). The schematic representation of the GST-fusion polypeptides, GST-3-90, GST-3-46, and GST-3-8, which were constructed from three cDNA clones, 3-90, 3-46, and 3-8, respectively, is shown in (B). These cDNA clones were selected by the yeast two-hybrid screening of the human B cell cDNA library (B).

EBNA-1(ΔGA) proteins in the absence of B cell lysates. However, it should be noted that the binding of the EBNA-1(ΔGA) proteins to the NPI-1 polypeptides was considerably weaker than the EBNA-1 proteins from the Raji cells; this less stable interaction might be due to the absence of B cell proteins and/or might be because of plausible less or different phosphorylation of EBNA-1(ΔGA), as discussed later.

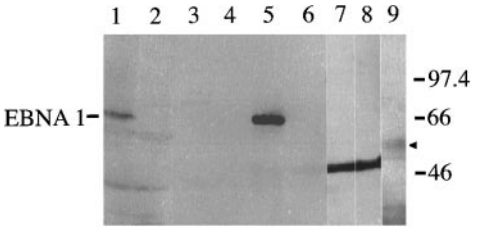

FIG. 2. *In vitro* binding of GST-fusion proteins of cDNA clone 3-90 to EBNA-1 in Raji cell lysates. GST-3-90-fusion proteins bound to glutathione-Sepharose 4B beads were incubated with EBNA-1 proteins in B cell lysates. Lane 1, EBNA-1 proteins in Raji cell lysates detected with anti-EBNA-1 monoclonal antibody EN2002; lane 2, reaction of DG75 cell lysates with the monoclonal antibody EN2002 as negative control; lane 3, GST proteins as negative control were bound to glutathione-Sepharose 4B, incubated with Raji cell lysates, eluted by adding glutathione, and assayed by Western blotting with the antibody EN2002; lane 4, GST proteins bound to glutathione-Sepharose 4B were incubated with cell lysates of EBV-negative B cell line DG75, eluted with glutathione, applied to Western blotting using the antibody EN2002; lane 5, GST-3-90 fusion proteins were bound to glutathione-Sepharose 4B, incubated with Raji cell lysate, eluted with glutathione and analyzed by immunoblotting with the antibody EN2002; lane 6, GST-3-90-fusion proteins were incubated with DG75 cell lysate, then assayed as described for lane 5; lane 7, GST-3-90 fusion proteins bound on glutathione-Sepharose 4B were incubated with Raji cell lysate, eluted, assayed by immunoblotting with the anti-NPI-1 rabbit antibody; lane 8, GST-3-90 fusion proteins bound on glutathione-Sepharose 4B were incubated with DG75 cell lysate, followed by the assay as noted for lane 7. Lane 9, EBNA-1(ΔGA) polypeptides synthesized in *E. coli* were incubated with glutathione-Sepharose 4B bead-bound GST-3-90 fusion proteins, eluted with glutathione, and applied to immunoblotting with the EN2002 antibody.

Another cDNA clone was Rch1, and its GST-fusion polypeptides interacted with endogenous EBNA-1 proteins in B cells

The base sequence of another cDNA clone prepared from a yeast two-hybrid positive colony, the cDNA clone 3-46, was identical to the 566- to 1230-bp region of another member of the karyopherin α family, Rch1(hSRP1α) [GenBank Accession No. U28386; identities = 659/665 (99%)]. In support of these sequence data, the GST-fusion polypeptides containing another cDNA clone 3-46, referred to as GST-3-46 fusion (Fig. 1B), positively reacted with anti-Rch1 rabbit antibodies as shown in Fig. 3A, lane 5. The protein interaction assay on the glutathione beads demonstrated that the GST-3-46 polypeptides bound to the Raji EBNA-1 proteins as shown in Fig. 3A, lanes 3 and 4. The interaction of Rch1 with EBNA-1 was reported earlier from the two laboratories; Fischer *et al.* (1997) examined the association upon *oriP* of the two proteins, testing a direct role of Rch1 on the EBV DNA replication, and Kim *et al.* (1997) revealed that EBNA-1 impeded formation of large protein complexes of Rch1 with cellular proteins. In their *in vitro* interaction tests, however, HA-tagged EBNA-1 polypeptides, which were translated *in vitro,* or HeLa S3 cells (Kim *et al.,* 1997), an epitheloid cell line established from cervical carcinoma and carries subgenomic fragments of human papilloma virus type 18 DNA (Meissner 1999), and recombinant EBNA-1 polypeptides synthesized in insect cells (Fischer *et al.,* 1997) were examined. Our results have demonstrated the interaction of Rch1 with EBNA-1 proteins endogenous in the Raji B cell line by the direct biochemical affinity assay in conjunction with Western blot analysis (Fig. 3A). The discrepancy between our results and the far-Western blot analysis, in which Rch1 did not interact with EBNA-1 proteins from B cells (Fisher *et al.,* 1997), seems to be due to a higher detectability by the GST-affinity analysis of a low concentration of proteins.

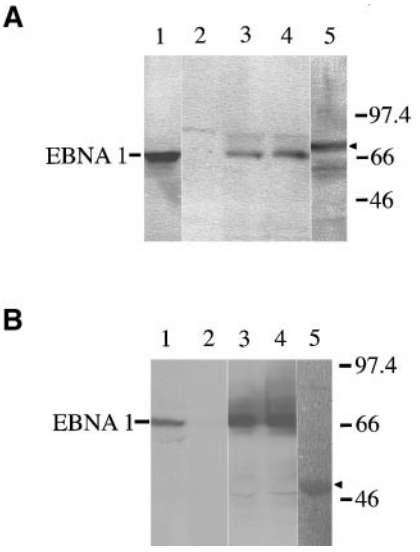

FIG. 3. Binding of GST-fusion proteins of cDNA clones 3-46 and 3-8 to EBNA-1 proteins in Raji cell lysates. (A) GST-3-46. Lane 1, detection with the anti-EBNA-1 monoclonal antibody EN2002 of EBNA-1 in Raji cell lysate; lane 2, Raji cell lysates were incubated with GST proteins as negative control that were bound on glutathione-Sepharose 4B beads beforehand, eluted with glutathione, and analyzed by Western blotting with the monoclonal antibody EN2002; lanes 3 and 4, Raji cell lysates were incubated with GST-3-46 polypeptides, which were bound on glutathione-Sepharose 4B beads, eluted, and analyzed by immunoblotting using EN2002. Two independently constructed GST-3-46 fusion polypeptides were separately analyzed in lanes 3 and 4; lane 5, GST-3-46 polypeptides were incubated with glutathione-Sepharose 4B, eluted, and detected by immunoblotting using the anti-Rch1 rabbit antibodies. (B) GST-3-8. Lane 1, direct detection of EBNA-1 proteins in Raji cell lysates as positive control with the anti-EBNA-1 monoclonal antibody EN2002; lane 2, Raji cell lysates were incubated with glutathione-Sepharose 4B bead-bound GST proteins as negative control, eluted with glutathione from the beads, and analyzed by Western blotting with the antibody EN2002. Lanes 3 and 4, Raji cell lysates were incubated with GST-3-8 fusion polypeptides that were bound to glutathione-Sepharose 4B beads, eluted from the beads, and analyzed by Western blotting with EN2002. Twofold cell lysates were added in the incubation mixture that was applied on lane 4 than that on lane 3; lane 5, GST-3-8 polypeptides were incubated with glutathione-Sepharose 4B, eluted with glutathione, and detected by immunoblotting using the mouse anti-p32/(gC/q-R) antibody.

The other cDNA clone was p32 and its GST fusion interacted with B cell endogenous EBNA-1

The base and deduced amino acid sequence of clone 3-8 agreed with that of 359–903 nucleotides (identity 88%) and 94–275 amino acids (identity 100%) of gC1q-R (EMBL X75913), that of 269–813 nucleotides (identity 88%) and 90–271 amino acids (identity 100%) of SF2p32 (GenBank M69039), and that of 91–272 amino acids (identity 100%) of TAP (Tat-associated protein) (Yu *et al.,* 1995). This cellular protein, which is known by various names, is referred to as p32 hereafter. It turned out that sequences of 32 of all 36 cDNA clones obtained using the Gly–Gly–Ala repeat-deleted bait conformed with p32. Further confirmation that the cDNA 3-8 is a p32 clone was obtained from the positive reaction of the GST-fusion polypeptides of clone 3-8, referred to as GST-3-8, (Fig. 1B), to the anti-p32 (gC1q-R) mouse antibody 60.11 (Fig. 3B, lane 5). The retention assay of the GST-3-8 polypeptides in a similar fashion indicated an interaction with the Raji endogenous EBNA-1 proteins, too (Fig. 3B, lanes 3 and 4). The region of p32 in the GST-3-8 is shorter than the mature form of p32 (aa 74–282) (Honore *et al.,* 1993), indicating that the amino-terminal 16 aa (aa 74–89, SF2p32 sequence) and carboxyl end 11 aa (aa 272–282, SF2p32) are not required for the interactions with EBNA-1 proteins. Furthermore these results have confirmed that EBNA-1 proteins endogenous in Raji cells bind *in vitro* to p32 proteins, whose coimmunoprecipitation with recombinant EBNA-1 proteins synthesized in the simian Cos-1 and human 293 cells, which were established by transformation of kidney cells with SV40 and adenovirus type 5, respectively, were previously reported (Chen *et al.,* 1998; Wang *et al.,* 1997).

Coimmunoprecipitation of EBNA-1 and NPI-1 proteins endogenous in B cells

To determine whether EBNA-1 interacts with endogenous NPI-1 in B lymphocytes, coimmunoprecipitation assay was employed using the Raji cell lysates. Proteins were coprecipitated with the anti-EBNA-1 monoclonal antibody EN2002 and examined by Western blot analysis with the rabbit anti-NPI-1 antibodies. A very broad band or doublet of 50–60 kDa in agreement with the NPI-1 double bands (Kohler *et al.,* 1997; Nadler *et al.,* 1997) was detected (Fig. 4, lane 1), indicating EBNA-1 binding to this NLS adapter protein *in vivo*. EBNA-1 proteins were detected using the two different anti-EBNA-1 mouse monoclonal antibodies, EN2002 and OT1x (Fig. 4, lanes 2 and 3). In the analyses of the cell lysate immunoprecipitates from the cell lysates by the anti-NPI-1 antibodies provided by Dr. P. Palese, two faint bands were detected with the anti-EBNA-1 EN2002 monoclonal antibodies: one at 66 kDa corresponding to the full-size EBNA-1 and another at 45 kDa, possibly its proteolytic product (data not shown). This low recovery of EBNA-1 with the anti-NPI-1 antibodies is considered to be due to their lower reactivity to the protein complexes or destabilization of the EBNA-1–NPI-1 complexes by the antibodies.

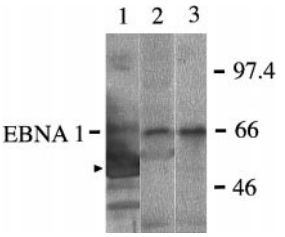

FIG. 4. Coimmunoprecipitation of endogenous NPI-1 and EBNA-1 proteins from Raji cell lysates with the anti-EBNA-1 monoclonal antibodies. Lane 1: Raji cell lysates were incubated with the anti-EBNA-1 monoclonal antibody EN2002 at 4°C for 1 h, protein G beads were added, and mixed gently at 4°C overnight, and the antigen–antibody complexes were eluted from protein G beads. The eluted proteins were analyzed by Western blotting using rabbit anti-NPI-1 antibody; lane 2: Raji cell EBNA-1 proteins were detected with the anti-EBNA-1 monoclonal antibody EN2002, lane 3, Raji cell EBNA-1 proteins were detected with the anti-EBNA-1 monoclonal antibody OT1x.

We also performed confocal microscopy analyses of Raji cells using the anti-NPI-1 antibodies obtained from Dr. P. Palese and from Dr. Y. Yoneda, the anti-EBNA-1 monoclonal antibody OT1x and the anti-EBNA-1 rat monoclonal antibody 2B4-1. NPI-1 proteins were stained in the cytoplasm of all the examined cells and in the nuclei of <10% in number. However, it was difficult to assess colocalization of EBNA-1 and NPI-1 proteins in the cells due to the too low immunofluorescent staining of EBNA-1 because of the antibodies' insufficient affinity to antigens fixed for immunofluorescence analysis (not shown).

DISCUSSION

EBNA-1 is the sole viral protein essential for the retention of EBV plasmid DNAs, which replicate once per cell cycle and are maintained at stable copy numbers per cell (Kieff, 1996; Rickinson and Kieff, 1996). Recently, it was demonstrated that EBNA-1 is not indispensable in the long-assumed replication of plasmids with *oriP* but is required for prevention of the rapid elimination of *de novo* synthesized *oriP* plasmids (Aiyar *et al.,* 1998). Moreover EBNA-1 is considered to mediate EBV plasmid partitioning to cell chromosomes (Marechal *et al.,* 1999). EBNA-1 is needed for efficient and stable latent EBV infection of B cells (Lee *et al.,* 1999), and the latent origin of replication of EBV is consistent with a stochastic model for DNA synthesis initiation (Kirchmaier and Sugden, 1998). EBNA-1 homodimers bind directly and specifically to the two sites: the family of repeats (FR) and the dyad symmetry element (DS) in *oriP* (Inoue *et al.,* 1991; Chen *et al.,* 1993; Kieff, 1996), linking the EBV DNA (Mackey *et al.,* 1995) and Qp (Sung *et al.,* 1994). EBNA-1

has been considered to play its roles through interaction with a cellular protein(s).

Cellular proteins that interact with EBNA-1

We suspect the activity(ies) and its subcellular/subnuclear localization of this multifunctional nuclear protein, whose mRNA expression is regulated by a cell cycle with no detectable change in protein levels during latency 1 (Davenport and Pagano, 1999), might be modulated by cellular proteins. These working hypotheses led us to search for cellular proteins which interact with EBNA-1 proteins employing a yeast two-hybrid system.

In the present study, we have demonstrated the interaction of EBNA-1 with NPI-1 protein. In addition this study exhibited that endogenous EBNA-1 proteins in the Raji B cell line of BL origin and latency type III bound to Rch1, whose interactions with baculovirus-expressed recombinant or hemagglutinin-tagged EBNA-1 polypeptides using different types of cells were reported earlier (Fisher *et al.,* 1997; Kim *et al.,* 1997). Our results confirmed that EBNA-1 endogenous in Raji cells interacted with p32, whose coimmunoprecipitation with recombinant EBNA-1 polypeptides in the cell lines of a different cell type established by viral transformation and whose colocalization with tagged EBNA-1 in Vero cells were previously reported (Chen *et al.,* 1997; Wang, Y. *et al.,* 1997).

Significance of interactions of EBNA-1 with NPI-1 and Rch1

What does the interaction of EBNA-1 with these two discrete NLS adapter proteins, NPI-1 and Rch1, suggest? First, EBNA-1 protein intriguingly interacts with these two NLS adaptors, which share 44.8% amino acid sequence homology (Weis *et al.,* 1995), implying its trafficking to the nucleus mediated by both of these proteins. This might suggest not only conceivable redundancy of a gene or protein of an essential function such as the nuclear transport but also regulation of the import system. In contrast to EBNA-1, nuclear import of Stat1 was mediated by NPI-1, not by Rch1 (Sekimoto *et al.,* 1997). DNA helicase Q1/RecQL interacted with Rch1 and Qip1, the third human karyopherin α, but not with NPI-1 (Seki *et al.,* 1997). These results suggest differential interactions of the NLS adapters/receptors to different karyophilic proteins. Interestingly, at least two more karyopherin α's have been shown to be expressed in human tissues (Kohler *et al.,* 1997; Malik *et al.,* 1997; Takeda *et al.,* 1997; Nachury *et al.,* 1998). On the other hand, influenza A virus nucleoprotein, NP, has the two overlapping nonconventional NLSs, SxGTKRSYxxM for binding to NPI-1 and TKRSxxxM for binding to Rch1 (Wang *et al.,* 1997). Interestingly, in the presence of HeLa cell cytosol, Rch1 bound to SV40T antigen, whereas NPI-1 and Qip1 bound poorly (Miyamoto *et al.,* 1997); in contrast, both NPI-1 and Rch1 strongly bound to SV40T antigen in the presence of lysates of Raji or Jurkat cells (Nadler *et al.,* 1997). NPI-1 binds to NLSs of Myc, HSV-1 ICP8 in the cytosolic extract of Raji cells, but not to either of the NLSs in the extract of Jurkat cells (Nadler *et al.,* 1997). These indicate that recognition of the NLSs by the adaptors is dependent on the cell type and modulated by cytosolic proteins (Nadler *et al.,* 1997). From this viewpoint, it is intriguing that the interaction of Rch1 with EBNA-1 required not only the unique NLS of EBNA-1, KRPRSPSS at aa 379–386 (Ambinder *et al.,* 1991), but also its upstream, aa 1–90, and downstream, aa 327–477 (Kim *et al.,* 1997) components. It is also noteworthy that a sequence of lys-arg in the NLS is shared between EBNA-1 and these proteins because the critical role of the lys-arg residues for NLS has been delineated by the X-ray crystallographic analysis of a tandem array of armadillo repeats in the yeast karyopherin α (Conti *et al.,* 1998).

Second, multiple karyopherin α's have recently been suggested to be expressed in one cell type and differentially in different cell types (Kohler *et al.,* 1997). The two differentially expressed karyopherin α's in leukocyte cell lines could be induced by cell activation (Nadler *et al.,* 1997). In contrast to Rch1, NPI-1 was at higher levels in the Raji and Jurkat cell lines than in the HeLa and several other cell lines (Kohler *et al.,* 1997). Thus EBNA-1's nuclear import might be more dependent on NPI-1 in B and T lymphocytes.

Third, function or activities of EBNA-1 proteins might be controlled through the nucleocytoplasmic trafficking and the localization pattern within the nucleus; diffused and granular localization patterns of EBNA-1 mutant proteins in the nuclei were described (Polvino-Bodnar *et al.,* 1988). Nuclear localization of EBNA-1 proteins has been well documented, but in a small number of EBV latently infected cells, EBNA-1 is detected in the cytoplasm, which usually is regarded as nuclear leakage during the experimental process (Petti *et al.,* 1990). It is intriguing that Wang *et al.* (1997) reported that full-length cytoplasmic p32 proteins translocated nuclear EBNA-1 into the cytoplasm. The actual function of p32, however, remains to be carefully elucidated, because it has recently been revealed that p32 has a new fold, giving a high negative charge density on one side of its trimer (Jiang *et al.,* 1999), is localized mainly in the mitochondrial matrix (Matthews *et al.,* 1998), and interacts with a very wide variety of cellular and viral proteins, namely ASF/SF2 splicing factor, lamin B receptor protein (p58), gC1q-R, TFIIB, hyaluronic acid, kininogen, vitronectin, HIV rev and tat, HSV-1 ORF P, EBNA-1, and Ad2 core protein V (Bruni and Roizman, 1996; Wang *et al.,* 1997; Chen *et al.,* 1998; Matthews and Russell, 1998). It, however, seems noteworthy that p32 is considered to be involved in nucleus–mitochondrion interactions (Matthews and Russell, 1998). On the other hand, it has recently been reported that accumulation rate and concentration of SV40 T an-

tigen in the nucleus were regulated by phosphorylation in the vicinity of the NLS (Jans, 1995). EBNA-1 is phosphorylated at the cluster of serines at aa 380–397 (Polvino-Bodnar *et al.,* 1988) that overlaps with its NLS defined by Ambinder *et al.* (1991), as we indicate in Fig. 1A by combining the data in the three papers (Hearing and Levine, 1985; Polvino-Bodnar *et al.,* 1988; Polvino-Bodnar and Schaffer, 1992). In addition, it is noteworthy that the aa 380–397 region has the serine-rich QSSSGSP stretch that immediately follows the NLS. Moreover, Kim *et al.* (1997) have indicated that the amino-terminal and central domains encompassing the EBNA-1 NLS contribute to its stable interaction with Rch1. In fact, the binding of EBNA-1(ΔGA) synthesized in *E. coli* to NPI-1 was weaker in comparison to Raji EBNA-1 (Fig. 2), although it might be explained as by the absence of other cellular proteins as described above. Thus the nuclear trafficking of EBNA-1 proteins might be controlled by its phosphorylation.

Finally, Rch1 (Rag cohort) is the protein initially defined as a major cell factor that interacted with the V(D)J recombinase-activating gene RAG-1, expression of which was restricted to immature lymphoid cells (Cuomo *et al.,* 1994). On the one hand, the human homolog of the yeast SRP1 that interacted with RAG-1 (Cortes *et al.,* 1994) is identical to the NPI-1 identified as a cellular protein that interacted with NP of influenza A virus (O'Neill and Palese, 1995); we have confirmed their perfect homology using the sequences in the former report (Cortes *et al.,* 1994) and the latter one, i.e., GenBank S75295 (O'Neill and Palese, 1995). On the other hand, EBNA-1 induced expression of RAG-1 and RAG-2 (Srinivas and Sixbey, 1995). Taken together, the transport of RAG-1 and its inducer EBNA-1, both mediated by the same pair of karyopherin α proteins, might efficiently enhance V(D)J recombinase activity; this could result in chromosome translocation, eliciting cytogenetic alterations associated with EBV infection such as *c*-myc protooncogene translocation into an immunoglobulin locus (Haluska *et al.,* 1986).

Perspectives

It is consistent with the recently clarified function of EBNA-1 for the retention, not for the synthesis, of EBV DNA (Aiyar *et al.,* 1998; Lee *et al.,* 1999; Shire *et al.,* 1999) that no known cellular protein directly involved in DNA duplication has been detected as an EBNA-1 binding factor by our protein interaction-trap system with either of the two different baits used in our study. The dual recognition of the unique NLS of EBNA-1 and its predicted nuclear transport by NPI-1 and Rch1, which might be differentially controlled by phosphorylation and cellular factors, is considered to facilitate possible modulation of subcellular localization and activities of the multifunctional EBNA-1 proteins.

MATERIALS AND METHODS

Antibodies

Mouse monoclonal antibody EN2002 against the truncated EBNA-1 polypeptide aa 451–641 was generated earlier in our laboratories (to be published elsewhere). Anti-EBNA-1 mouse monoclonal antibody OT1x (Chen *et al.,* 1993) and rat monoclonal antibody 2B4–1 (Grasser *et al.,* 1994) were kindly provided by Dr. Y. Middeldorp (Organon Teknika, The Netherlands) and Dr. F. A. Grasser (Universitatskliniken, Homburg, Germany) respectively. The anti-gC1q-R (p32) monoclonal antibody 60.11 was a kind gift from Dr. B. Ghebrehiwet (State University of New York at Stony Brook). The anti-Rch1 rabbit antibody was kindly provided by Drs. M. V. Nachury and K. Weis (University of California, San Francisco). Two rabbit antibodies against NPI-1 were provided kindly by Dr. P. Palese (Mount Sinai School of Medicine, New York) and Dr. Y. Yoneda (Osaka University, Japan).

Protein–protein binding assay with GST-fusion polypeptides and glutathione beads

GST-fusion polypeptides from the yeast two-hybrid selected cDNA clones were bound to glutathione-Sepharose 4B beads (Pharmacia, Sweden). Proteins of interests in Raji cells or DG75 cells, which were grown at 37°C in a CO_2 incubator, were added to the GST-fusion protein-bound glutathione-Sepharose 4B and mixed at 4°C. The bound proteins were eluted with glutathione, resolved on an 8% SDS–polyacrylamide gel, and electrotransferred to nitrocellulose membrane (Bio-Rad). The membrane was then incubated with the antibodies indicated in the text, and proteins were detected with the alkaline phosphatase detection system (Bio-Rad).

Coimmunoprecipitation assay

Cell lysates of interest were incubated with antibodies at 4°C for 1 h. Protein G–agarose beads were added to the reaction and mixed at 4°C, and subsequently the absorbed immunoprecipitates were eluted from protein G.

Western blot analyses

Proteins were separated on an 8% SDS–polyacrylamide gel at 15 V/cm and electrotansferred to nitrocellulose membrane. Subsequently, the membrane was incubated with indicated primary antibodies at 37°C for 90 min, followed by the secondary alkaline phosphatase-conjugated antibodies (Bio-Rad), and treated with the immunoblotting detection reagent (Bio-Rad).

Cells and lysates

The EBV-negative DG75 human B cell line established from Burkitt' lymphoma was a kind gift from Dr. G. Klein

(Karolinska Institute, Sweden) and the EBV latently infected B cell line Raji, also BL-derived, was purchased from ATCC (Rockville, MD). The B cells were cultured in RPMI medium containing 10% fetal bovine serum, 100 μg/ml kanamycin, 25 μg/ml amphotericin B (Boehringer-Manheim, Germany) at 37°C in a 5% CO_2 humidified incubator. Cells were centrifuged, washed, and resuspended in a cell lysate preparation buffer (10 mM Tris–HCl, pH 7.5, 25% glycerol, 2 mM Mg_2Cl, 0.1 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 0.1% NP-40, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 20 μg/ml phosphoramidon). Subsequently, harvested cells were ultrasonicated and centrifuged, and the supernatant was used.

Preparation of glutathione-*S*-transferase fusion proteins

For construction of GST-fusion polypeptides, DNA fragments from two-hybrid screening positive cDNA clones were inserted in the polylinker segment of PGEX-5X-1 (Amersham Pharmacia Biotech). The expression vectors were used to transform the *E. coli* BL21(DE3) strain. The transformed *E. coli* cells were cultured, harvested after IPTG induction, and ultrasonicated in PBS buffer containing 0.5% *N*-lauroylsarcosine (Nakarai Tesque, Japan), 5% Triton X-100 (Nakarai Tesque), 0.5 μg/ml aprotinin (Boehringer-Mannheim), 0.5 μg/ml leupeptin (Boehringer-Mannheim), 0.7 μg/ml pepstatin (Boehringer-Mannheim), 20 μg/ml phosphoramidon (Boehringer-Mannheim), and 5 mM dithiothreitol on ice with a MICROSON ultrasonic cell disruptor (Heat Systems). The disrupted cells were then spun at 14,000 rpm at 5°C for 10 min, and the supernatants were used. The truncated EBNA-1 polypeptide lacking the Gly–Gly–Ala repeats, EBNA-1(ΔGA), was generated from p205 that lacks the Gly–Gly–Ala repeated region of EBNA-1 (Fig. 1A) and was provided from Dr. B. Sugden (Wisconsin University) (Yates *et al.,* 1985). The truncated EBNA-1 sequence was amplified by PCR using the primers, 5′-gccggatcccatgtctgacgaggggccag-3′ and 5′-gccaagcttttattctttagtgcgggggga-3′, cleaved with *Hin*dIII and *Bam*HI, followed by insertion into the pRSETB vector (Invitrogen, The Netherlands). The expression vector pRSETB-EBNA-1(ΔGA) (Fig. 1A) was used to transform the *E. coli* strain BL21(DE3).

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